Potential pathogenic role of aggregative-adhering *Corynebacterium diphtheriae* of different clonal groups in endocarditis

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Invasive diseases caused by *Corynebacterium diphtheriae* have been described increasingly. Several reports indicate the destructive feature of endocarditis attributable to nontoxigenic strains. However, few reports have dealt with the pathogenicity of invasive strains. The present investigation demonstrates a phenotypic trait that may be used to identify potentially invasive strains. The study also draws attention to clinical and microbiological aspects observed in 5 cases of endocarditis due to *C. diphtheriae* that occurred outside Europe. Four cases occurred in female school-age children (7-14 years) treated at different hospitals in Rio de Janeiro, Brazil. All patients developed other complications including septicemia, renal failure and/or arthritis. Surgical treatment was performed on 2 patients for valve replacement. Lethality was observed in 40% of the cases. Microorganisms isolated from 5 blood samples and identified as *C. diphtheriae* subsp mitis (N = 4) and *C. diphtheriae* subsp gravis (N = 1) displayed an aggregative adherence pattern to HEp-2 cells and identical one-dimensional SDS-PAGE protein profiles. Aggregative-adhering invasive strains of *C. diphtheriae* showed 5 distinct RAPD profiles. Despite the clonal diversity, all 5 *C. diphtheriae* invasive isolates seemed to display special bacterial adhesive properties that may favor blood-barrier disruption and systemic dissemination of bacteria. In conclusion, blood isolates from patients with endocarditis exhibited a unique adhering pattern, suggesting a pathogenic role of aggregative-adhering *C. diphtheriae* of different clones in endocarditis. Accordingly, the aggregative-adherence pattern may be used as an indication of some invasive potential of *C. diphtheriae* strains.

Key words: Aggregative adherence; *Corynebacterium diphtheriae*; Endocarditis; HEp-2 cells; Random amplified polymorphic DNA


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Introduction

Invasive diseases add new aspects to the infectious processes caused by *Corynebacterium diphtheriae*, and identify this species as an emerging pathogen. In the early years of the new millennium, infective endocarditis still proves to be difficult to diagnose and is associated with a high death rate (21-41%) worldwide (1,2). Infective endocarditis due to *C. diphtheriae* is perhaps more common than supposed and on occasion may be an aggressive fatal disease (3). Few reports have dealt with the microbiological aspects of community-acquired endocarditis caused...
by *C. diphtheriae* (4-6). Entry of *C. diphtheriae* by invasive processes was described as being caused by percutaneous trauma, skin (7,8) and throat colonization (9). Unlike classical diphtheria, invasive disease caused by *C. diphtheriae* affects both vaccinated and non-vaccinated persons, and is mostly induced by nontoxigenic isolates (10). Diphtheria epidemics are likely to spread with a clonal character (11). Likewise, systemic diseases caused by *C. diphtheriae* have been related to invasive clones (7,12,13).

In addition to host factors, bacterial virulence determinants may contribute to the outcome of invasive infections. Microbial adhesive properties may contribute to the spread and outcome of invasive processes (14). Despite the destructive feature of invasive infections, the pathogenicity aspects that favor the invasive ability of certain *C. diphtheriae* strains remain unclear (10,15). The ability to survive within cultured epithelial cells was observed for *C. diphtheriae* strains isolated from throat and blood. The blood isolate showed a higher percentage of adherence and internalization compared with throat isolates (5). Further study showed that all sucrose fermenting and non-fermenting strains isolated from throat and skin lesions exhibited localized and diffuse adherence patterns, respectively (16). However, the binding properties of *C. diphtheriae* strains related to invasive disease have not yet been investigated. Moreover, identifying organisms belonging to invasive clones, including locations where diphtheria is rarely encountered, may provide valuable information and allow timely preventive measures.

In addition to describing clinical and microbiological characteristics that illustrate the destructive features of endocarditis due to *C. diphtheriae*, this study was undertaken to assess the patterns of adherence to HEp-2 cells of endocarditis-associated *C. diphtheriae* strains representative of five different clones.

### Material and Methods

#### Bacterial strains and growth conditions

Five *C. diphtheriae* strains isolated from blood samples of patients with endocarditis were used to illustrate specific biological aspects of invasive disease and to evaluate phenotypic methods that could be used to discriminate potentially invasive strains (Table 1). Microorganisms were recovered from clinical specimens routinely submitted for culturing to the Bacteriology Laboratory of Hospital Universitário Pedro Ernesto, HUPE (LABAC) and the Laboratory of Diphtheria and Non-diphtheria Corynebacterial Infections at Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro (UERJ), Rio de Janeiro, RJ, Brazil.

For phenotypic and genotypic analysis, the microorganisms were grown in trypticase soy broth (Difco Lab., USA) or agar medium for 48 h at 37°C. Two toxigenic strains (CDC-E8392 from Centers for Disease Control, USA, and 241 from Rio de Janeiro, Brazil, isolated from the respiratory tract of patients with diphtheria) were used for comparison.

#### Biochemical identification and toxigenicity test

Identification, biotype, and toxigenicity determination were performed by conventional microbiological methods as described elsewhere and by the API Coryne System (bioMérieux, France). The toxigenicity test was performed by the Elek assay (6,15,17,18).

**HEp-2 adherence assays**

Adherence patterns to HEp-2 of *C. diphtheriae* blood isolates were assayed by methods previously described (16). Adherence assays were performed within semi-confluent HEp-2 cells grown on circular coverslips in DMEM (Sigma, USA) supplemented with 5% fetal calf serum (Gibco-BRL, USA), 50 µg/mL gentamicin, 2.5 µg/mL amphotericin B and 0.5% L-glutamine at 37°C in a 5% CO<sub>2</sub> atmosphere. Microorganisms were washed twice in 10 mM phosphate-buffered saline (PBS, pH 7.2) and resuspended in DMEM to a concentration of 10<sup>7</sup> colony-forming units per mL, and used in adherence assays (3-h incubation). Giemsa-stained coverslips were examined by bright field microscopy.

**SDS-PAGE analysis**

Protein profiles of crude extracts of bacterial strains were obtained after bacterial cell lysis by treatment with 5 mg/mL lysozyme (Sigma) in PBS for 2 h at 37°C. Thereafter, protein samples were submitted to treatment with sample buffer [0.5 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% β-mercaptoethanol, and 0.001% bromophenol blue] and heating at 100°C for 15 min. Total crude cell lysates were cleared by centrifugation at 14,000 g for 5 min, and submitted to electrophoresis on 10% polyacrylamide gels in the presence of SDS (SDS-PAGE). The protein profiles were observed after staining with Coomassie brilliant blue R250 (Sigma) (19).

**RAPD analysis**

RAPD was performed using the Ready-to-go RAPD Kit (Pharmacia Biotech, USA), according to manufacturer instructions on a Minicycler PTC-100 Programmable Thermal Controller (MJ Research, USA) (20). Amplified products were electrophoresed on a 2% agarose gel for 3 h at 120 V. Gels were stained with ethidium bromide and the
amplicons were analyzed based on the unweighted pair group method with arithmetic mean (UPGMA) (21).

**Results and Discussion**

Clinical and microbiological features related to *C. diphtheriae* strains isolated from patients with native-valve endocarditis are presented in Table 1. Four strains (HC01, HC02, HC03, HC05) were identified as *C. diphtheriae* subsp mitis and one (HC04) as *C. diphtheriae* subsp gravis. All strains were toxigenic by the Elek assay.

We have found the incidence of infective endocarditis in the literature to be generally higher in males than in females (2:1), and the average age group affected is in the fifth decade (1). In contrast, the highest number of *C. diphtheriae* endocarditis in our community was found in children less than 18 years of age rather than in adults over 50 years of age (4:1), and in females more than in males (4:1). No cases were reported among children less than 5 years of age. The source of bacteremia was not precisely determined for all 5 cases. However, predisposing conditions to an increased risk of acquiring endocarditis (22), such as congenital heart disease and major dental treatment, were observed for patients HC03 and HC05, respectively. Patient HC02 experienced an episode of non-exudative pharyngitis 2 weeks before the onset of infection. Nevertheless, skin lesions were not seen in any patient. The preferred sites of attack of *C. diphtheriae* endocarditis isolates were mitral valves (80%). Complications observed in all five episodes of endocarditis included septicemia (N = 5), intracardiac abscess (N = 1), mycotic aneurysm (N = 1), peripheral (N = 2) and CNS emboli (N = 1), microaneurysm with brain hemorrhage (N = 2), arthritis (N = 2), and acute renal failure (N = 3). Surgical treatment was performed for valve replacement for patients HC02 and HC05. Patients HC03, HC04, and HC05 were submitted to antibiotic and serum therapy. The mortality rate (40%) due to infective endocarditis caused by *C. diphtheriae* was higher than that previously reported in the literature (27%) (23).

*C. diphtheriae* endocarditis isolates exhibited an aggregative-like adherence pattern, characterized by clumps of bacteria with a "stacked-brick" appearance that were attached to the surfaces of cultured epithelial cells and exposed areas of the glass slide among epithelial

**Table 1. Clinical aspects of patients with endocarditis and microbiological properties of Corynebacterium diphtheriae blood isolates.**

<table>
<thead>
<tr>
<th>Case/year</th>
<th>Gender/age/ childhood immunization*</th>
<th>Presumptive/ conclusive diagnosis</th>
<th>Complications</th>
<th>Prior heart defect</th>
<th>Heart valve destruction</th>
<th>Outcome</th>
<th>Microbiological properties b</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC01/1993</td>
<td>Female/ 14 years old/ yes</td>
<td>Sepsis/ endocarditis</td>
<td>Peripheral emboli of legs and arms; microaneurysm with brain hemorrhage</td>
<td>No</td>
<td>Mitral</td>
<td>Dead</td>
<td>Toxigenic <em>C. diphtheriae</em> subsp mitis</td>
</tr>
<tr>
<td>HC02/1999</td>
<td>Female/ 9 years old/ yes</td>
<td>Rheumatic fever/ endocarditis</td>
<td>Arthritis; myositis; valvar abscess; acute renal failure</td>
<td>No</td>
<td>Mitral</td>
<td>Valve replacement; alive</td>
<td>Toxigenic <em>C. diphtheriae</em> subsp mitis</td>
</tr>
<tr>
<td>HC03/2000</td>
<td>Female/ 9 years old/ yes</td>
<td>Meningitis/ endocarditis</td>
<td>Mycotic aneurism; acute renal failure</td>
<td>Congenital Mitral, tricuspid</td>
<td>Alive</td>
<td>Toxigenic <em>C. diphtheriae</em> subsp mitis</td>
<td></td>
</tr>
<tr>
<td>HC04/2003</td>
<td>Female/ 7 years old/ yes</td>
<td>Septic shock/ endocarditis</td>
<td>Arthritis; myositis; peripheral and CNS emboli; microaneurysm with brain hemorrhage</td>
<td>Congenital Mitral, aortic</td>
<td>Dead</td>
<td>Toxigenic <em>C. diphtheriae</em> subsp gravis</td>
<td></td>
</tr>
<tr>
<td>HC05/2005</td>
<td>Male/ 58 years old/ yes</td>
<td>Sepsis/ endocarditis</td>
<td>Weight loss; acute renal failure</td>
<td>No</td>
<td>Aortic</td>
<td>Valve replacement; alive</td>
<td>Toxigenic <em>C. diphtheriae</em> subsp mitis</td>
</tr>
</tbody>
</table>

*Absence of skin lesions. bNose and throat cultures were not performed. Toxin production evaluated by the Elek test. cMicrobiological description of bacterial strain published by Mattos-Guaraldi et al. (15). dPharyngitis without pseudomembrane formation two weeks before the onset of infection. ePatient submitted to prior dental surgery. fDTP, diphtheria, tetanus and pertussis.
Phenotypic and molecular typing methods have been used with varying success to distinguish between *C. diphtheriae* strains (26,27). Despite the fact that SDS-PAGE profiles are not considered to provide consistent results for the evaluation of clonality among bacterial strains, heterogeneity among protein profiles of *C. diphtheriae* has been used for the analysis of epidemic strains (28,29). In the present study, SDS-PAGE demonstrated *C. diphtheriae* endocarditis isolates characterized by a particular protein profile, as exemplified by the HC01 strain in Figure 2. However, the endocarditis isolates showed a protein profile distinct from those presented by *C. diphtheriae* 241 and CDC-E8392 strains isolated from patients with diphtheria. Thus, in addition to the aggregative pattern to HEp-2 cells,
the relationship among C. diphtheriae endocarditis isolates was verified by the expression of identical SDS-PAGE protein profiles.

RAPD-PCR can be reliably and reproducibly used for rapidly screening C. diphtheriae strains of the predominant epidemic clonal group (30). C. diphtheriae endocarditis isolates showed different amplification patterns by RAPD-PCR for all 6 sets of primers used, indicating diversity among C. diphtheriae invasive clones. The use of RAPD-PCR was successful for detecting minor differences in closely related toxigenic C. diphtheriae endocarditis strains. The primer 4 of RAPD-PCR kit was more discriminating for closely related toxigenic C. diphtheriae endocarditis strains. PCR was successful for detecting minor differences in invasive properties. The data also suggest the pathogenic role of aggregative-adhering C. diphtheriae in invasive disease.

Additional studies are necessary to characterize the full range of virulence factors of the aggregative-adhering C. diphtheriae and the molecular pathophysiology of the invasive illness caused by these strains. It has been demonstrated that E. coli strains, which present aggregative adherence, are able to form biofilms (31). This property enables aggregative-adhering E. coli strains to produce more prolonged diseases. The possibility that C. diphtheriae can also form biofilms (15) and the potential of this property to aggravate the clinical outcome of invasive disease merits investigation.

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